Characterizing the Distribution and Rates of Microbial Sulfate Reduction at Middle Valley Hydrothermal Vents

Characterizing the distribution and rates of microbial sulfate reduction at Middle Valley hydrothermal vents

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ABSTRACT

Few studies have directly measured sulfate reduction at hydrothermal vents, and relatively little is known about how environmental or ecological factors influence rates of sulfate reduction in vent environments. A better understanding of microbially mediated sulfate reduction in hydrothermal vent ecosystems may be achieved by integrating ecological and geochemical data with metabolic rate measurements. Here we present rates of microbially mediated sulfate reduction from three distinct hydrothermal vents in the Middle Valley vent field along the Juan de Fuca Ridge, as well as assessments of bacterial and archaeal diversity, estimates of total biomass and the abundance of functional genes related to sulfate reduction, and *in situ* geochemistry. Maximum rates of sulfate reduction occurred at 90°C in all three deposits. Pyrosequencing and functional gene abundance data reveal differences in both biomass and community composition among sites, including differences in the abundance of known sulfate reducing bacteria. The abundance of sequences for *Thermodesulfovibro*-like organisms and higher sulfate reduction rates at elevated temperatures, suggests that *Thermodesulfovibro*-like organisms may play a role in sulfate reduction in warmer environments. The rates of sulfate reduction presented here suggest that - within anaerobic niches of hydrothermal deposits - heterotrophic sulfate reduction may be quite common and can contribute to secondary productivity, underscoring the potential role of this process in both sulfur and carbon cycling at vents.

Keywords: Hydrothermal Vent/Microbial Ecology/Primary productivity/Sulfate Reduction

Subject Category: Geomicrobiology and microbial contributions to geochemical cycles

INTRODUCTION

Deep sea hydrothermal vent ecosystems are complex dynamic habitats characterized by steep gradients in temperature and geochemistry (Jannasch & Mottl, 1985). In these habitats, as hot hydrothermal fluid mixes with cold seawater, the precipitation of minerals creates large and complex hydrothermal chimney deposits. Within these permeable mineral structures, the continued mixing of chemically reduced, vent-derived fluids and oxidized seawater provides favorable conditions that support the growth of endolithic microbial communities (Schrenk *et al.* 2003)

Sulfide oxidation is considered to be one of the most important microbial chemosynthetic pathways at ridge ecosystems, as evidenced by the ubiquity of sulfide oxidizing *Epsilon*- and *Gammaproteobacteria* at ridge environments (Nakagawa *et al*. 2004; Huber *et al*. 2007; Nakagawa & Takai, 2008; Nakagawa *et al*. 2005; Campbell *et al.* 2006). To date, significantly less attention has been paid to the distribution and magnitude of sulfate reduction at vents, though sulfate reducing bacteria and archaea have frequently been isolated from deep sea hydrothermal environments (Houghton *et al*. 2007; Audiffrin *et al*. 2003; Alazard *et al*. 2003; Jannasch *et al*. 1988; Blöchl *et al*. 1997). Moreover, analyses of functional genes that express key proteins required for sulfate reduction suggest there is a high diversity of sulfate reducing organisms at vents, higher than predicted via 16S rRNA gene analyses alone (Nakagawa *et al*. 2004; Nercessian *et al*. 2005).

From a biogeochemical and bioenergetic perspective, both sulfide oxidation and sulfate reduction would be favored at hydrothermal vents, though to varying degrees as a function of environmental chemistry. Sulfide oxidation is most favorable when coupled to oxygen or nitrate

as an electron acceptor (Amend & Shock 2001). Around vents, sulfide is typically in µM to mM concentrations (Butterfield, et al. 1994; Butterfield et al. 1994), while oxygen and nitrate are around 110 and 40 µM respectively (Johnson *et al*. 1986). In contrast, sulfate reduction is highly favored in anoxic niches at vents, as it is in other marine anaerobic environments (Muyzer & Stams, 2008). Here, as in most marine systems, sulfate is abundant at 28 mM two to three orders of magnitude higher than oxygen At vents, sulfate reduction would occur in regimes where seawater-derived sulfate is still present but oxygen is absent, e.g. within hydrothermal vent deposits. Sulfate reducing microorganisms commonly use hydrogen and/or dissolved organic matter as electron donors, both of which are found within hydrothermal fluids (Lang *et al*. 2006; Cruse & Seewald, 2006). Sulfate reduction –as a function of its extent and magnitude- could readily influence the cycling of sulfur and sulfur isotopes, as well as carbon, within hydrothermal environments.

To date, studies have quantified rates of sulfate reduction in hydrothermal-influenced sediments (Weber & Jorgensen, 2002; Jorgensen *et al*. 1992; Elsgaard *et al.* 1994; Kallmeyer & Boetius, 2004; Elsgaard *et al*. 1994; Elsgaard *et al*. 1995) and isolated vent microorganisms (Hoek *et al*. 2003). In contrast to the numerous studies of sulfate reduction in marine sediments (Canfield 1989), studies of sulfate reduction in hydrothermal deposits are few (Bonch-Osmolovskaya *et al*. 2011), due in part to the challenges associated with sampling and studying the heterogeneous and consolidated sulfide deposits typical of hydrothermal vent chimneys.

Here we present rates of microbially mediated sulfate reduction from three distinct, active hydrothermal “chimneys” found in the Middle Valley field along the Juan de Fuca Ridge, as well as assessments of bacterial and archaeal diversity, estimates of total biomass and the

abundance of functional genes related to sulfate reduction, and *in situ* geochemistry. These analyses further our understanding of sulfate reduction (including rates, diversity and distribution of known sulfate-reducing microbes) in vent ecosystems. Moreover, they underscore the potential role of heterotrophic sulfate reduction in hydrothermal systems, and constrain their potential influence on both sulfur and carbon cycling,

METHODS

Geologic Setting and Sampling of hydrothermal deposits

Middle Valley (48°27'N, 128°59' W) is an intermediate spreading, axial rift valley, located along the Endeavor Segment of the Juan de Fuca Ridge in the Northwest Pacific ocean. Layers of continental-derived sediments characteristically cover Middle Valley, though the hydrothermal vents remain prominent above the sediments. Hydrothermal deposits were collected from 3 active hydrothermal spires during dive 4625 with the *HOV Alvin* (*R/V Atlantis* expedition AT15-67, July 2010) and brought to the surface in a sealed, temperature-insulated polyethylene box. Samples were recovered from actively venting sulfide deposits at Needles (48.45778, -128.709, 2412.212 m, T_{max}=123°C), Dead Dog (48.45603,-128.71, 2405.268 m, T_{max}=261°C), and Chowder Hill (48.455543, -128.709, 2398.257 m, T_{max} =261°C) vents. Once on board ship, samples were directly transferred to sterile anaerobic seawater and handled/processed using appropriate sterile microbiological techniques. Subsamples were immediately transferred to gastight jars (Freund Container Inc.), filled with sterile anaerobic seawater containing 2 mM sodium sulfide at pH 6, and stored at 4°C. Upon return to the laboratory, all samples were provided with fresh 2 mM sulfidic, anaerobic seawater every 8 to 12 weeks and were kept in the dark and 4°C prior to incubation.

Vent fluid volatile geochemistry via *in situ* mass spectrometry

In situ concentration of dissolved volatiles (H_2S, H_2, CO_2, O_2, and others.) were measured at each site with an *in situ* mass spectrometer (ISMS) as previously described (Wankel *et al*. 2011). Briefly, dissolved volatiles were quantified *in situ* by sampling vent effluent for up to 10 minutes, until partial pressures reached steady state (data was monitored in real time within the submersible). Concentrations were determined from empirically derived calibrations and validated by comparison with discrete samples collected using titanium gastight samplers.

Measuring sulfate reduction rates

Hydrothermal deposits were homogenized in a commercial blender (Xtreme™ blender, Waring Inc.) under a nitrogen atmosphere. Anaerobic homogenization was designed to minimize fine-scale geochemical and microbial heterogeneity and facilitate more accurate experimental replication. Hydrothermal homogenate (made up of both mineral deposit and interstitial fluid)) was aliquoted volumetrically (7.5 mL, ca. 29 g wet weight and ca. 20 g dry weight) into Balch tubes in an anaerobic chamber. The tubes were supplemented with 15 mL of sterile artificial vent fluid media designed to mimic the geochemical conditions within a sulfide deposit (pH 6, 14 mM SO_4^{2-}, 2.3 mM $NaHCO_3$, 1 mM H_2S, and 10 µM each of pyruvate, citrate, formate, acetate, lactate). Organic acid concentrations are comparable to those measured *in situ* (Lang *et al*. 2006). Sufficient $^{35}SO_4^{2-}$ was added to achieve 555 kBq (15 µCi) of activity. Due to technical difficulties with post processing methodology, shipboard incubations using fresh material were not successful. The data presented here were generated using samples that had been kept at 4 °C and refreshed with vent-like effluent (as described above) for one year. Samples were incubated anaerobically for 7 days at 4, 30, 40, 50, 60, 80 and 90°C. Controls for

sulfate reduction consisted of samples amended with 28 mM molybdate, a competitive inhibitor of sulfate reduction (Saleh *et al.* 1964; Newport & Nedwell, 1988). Six biological replicates were run for each treatment, and three biological replicates for each control. Upon completion, reactions were quenched with the injection of 5 mL 25% zinc acetate (which is ~20-fold more Zinc than the maximum sulfide concentration), and all samples were frozen at -20° C for further analysis.

To determine sulfate reduction rates, samples were thawed and the supernatant was removed and filtered through a 0.2 µm syringe filter. The crushed deposits that remained in the tube were washed three times with deionized water to remove any remaining sulfate. One gram (wet weight) of crushed deposit was analyzed via chromium distillation (see Supplemental Methods) and sulfate reduction rates (SRR) were calculated as in (Fossing & Jorgensen, 1989)using the following calculation.

$$SRR = \frac{nSO_4^{2-} \cdot a \cdot 1.06}{(a+A) \cdot t} \quad \text{Eq.1}$$

Where nSO_4^{2-} is the quantity (in moles) of sulfate added to each incubation (14 mM * 15 mL = 210 µmol), *a* is the activity (dpm) of the trapped sulfide, 1.06 is the fractionation factor between the sulfide and sulfate pools, *A* is the activity of the sulfate pool at the completion of the incubation and t is the incubation time (days). The rates are presented in units of nmol S g^{-1} day^{-1}.

DNA Extraction

Immediately prior to conducting the rate experiments, a subsample of homogenized hydrothermal deposit was removed and frozen at -80° C for molecular analysis. DNA was

extracted from this crushed deposit sample with a protocol modified from (Santelli *et al*. 2008). Subsamples were washed with 0.1 N HCl, followed by two rinses with a sterile solution containing 10 mM Tris (pH 8.0) and 50 mM EDTA. A known mass of material was added to PowerSoil beadbeating tubes(MoBio Laboratories, Carlsbad CA), incubated at 70**°**C for 10 minutes, and then amended with 200 ng of poly-A. Subsamples were subjected to beadbeating, followed by three cycles of freeze-thaw steps to further lyse cells. Nucleic acids were extracted using hot phenol (60**°**C for 3 min.), followed by two chloroform:isoamyl separations and precipitated with ethanol. DNA was resuspended in TE (pH 8.0) and quantified using the Qubit™ fluorometer (Life Technologies, Grand Island, NY).

Enumeration of gene abundance via quantitative PCR

Quantitative PCR (qPCR) was used to determine the abundance of bacterial and archaeal 16S rRNA genes. In addition, qPCR was used to enumerate the abundance of sulfate reducing prokaryotes by amplifying the adenosine 5‘-phosphosulfate reductase (*aprA*) gene with primers targeting sulfate reducing bacteria and archaea (Christophersen et al. 2011). Primers specific to bacterial dissimilatory sulfite reductase (*dsrA*) (Kondo et al. 2004) and *Deltaproteobacteria* 16S rRNA genes (Stults et al. 2001) provide alternate estimates of sulfate reducing bacteria populations. Quantification was performed in triplicate with the Stratagene MX3005p qPCR System (Agilent Technologies) using the Perfecta SYBR FastMix with low ROX (20 µL reactions, Quanta Biosciences, Gaitherburg, MD), specific primers and annealing temperatures (Table 1) and 10 ng of template gDNA. The temperature program for all assays was 94°C for 10 minutes, 35 cycles of 94°C for 1 minute, the annealing temperature for 1 minute (Table 1), extension at 72°C for 30 seconds and fluorescence read after 10 seconds at 80°C. Following amplification,

dissociation curves were determined across a temperature range of 55°C to 95°C. Ct values for each well were calculated using the manufacturer's software. Plasmids containing bacterial and archaeal 16S rRNA and functional gene inserts (amplified from *Arcobacter nitrofigulis* (ATCC 33309), *Methanosarcina acidovorans* and *Desulfovibrio vulgaris* Hildenborough (ATCC 29579/ NCIMB 8303/ AE017285) respectively) were used as standards for calibration (see Supplemental Methods for more detail).

Sequencing and Phylogenetic Analysis via 454 pyrosequencing

DNA samples were sequenced by Research and Testing Laboratory (Lubbock, TX) using 454 pyrotag methods similar to those described previously (Dowd *et al*. 2008). All samples were sequenced using a 454FLX instrument (Roche Inc.) with Titanium™ reagents. The resulting bacterial and archaeal 16S rRNA and *drsB* genes (primers in Table 1) datasets were analyzed via Mothur (Schloss *et al*. 2009). Sequences were trimmed, quality checked, aligned to the SILVA-compatible alignment database reference alignment (*dsrB* gene datasets were aligned to a *dsrB* gene database generated from the Ribosomal Database Project (RDP)), analyzed for chimeras, classified against the Greengenes99 database and clustered in to OTUs (see Supplemental Methods for more detail). Rarefaction curves were used to examine the number of OTUs as a function of sampling depth. Alpha diversity was assessed by generating values from the Chao1 richness estimators and the inverse Simpson diversity index.

Sequence Accession numbers

The 16S rRNA and *dsrB* gene sequences reported in this study have been submitted to Sequence Read Archive under the accession numbers SRX154520 through SRX154528.

RESULTS

Physical and geochemical characteristics of the study sites

The hydrothermal deposits sampled from Middle Valley were all relatively friable and were composed predominantly of anhydrite ($CaSO_4$, M. Tivey, pers. comm). Chowder Hill and Dead Dog had the highest observed venting fluid temperatures (measured *in situ* at 261°C), followed by Needles (123°C). *In situ* measurements of dissolved hydrogen sulfide (H_2S) revealed significant differences in hydrothermal fluid composition among hydrothermal deposits. Unfortunately the inline pH probe with the ISMS malfunctioned during the dive. Using previously reported pH values (Butterfield *et al*. 1994), Chowder Hill would have the highest *in situ* measurement of total sulfide (3.9 mM), followed by Dead Dog (2.2 mM), and Needles (0.59 mM) (Table 2). These concentrations are within the same magnitude of previously reported H_2S in focused vent fluids at Middle Valley (Butterfield *et al*. 1994). Chowder Hill did exhibit the highest *in situ* concentration of hydrogen (1.86 mM) followed by Dead Dog (1.66 mM) and Needles (~1.42 mM). These values are also consistent with previous studies (Cruse & Seewald 2006), as well as gastight samples collected and analyzed shipboard (M. Lilley, pers. comm).

Sulfate Reduction Rates

Among all samples, sulfate reduction was observed at temperatures between 4°C to 90°C (Figure 1). Maximal rates of sulfate reduction were observed between 88-90°C (2670 nmol g^{-1} day^{-1} at Needles, 1090 nmol g^{-1} day^{-1} at Chowder Hill, and 142 nmol g^{-1} day^{-1} at Dead Dog; Figure 1). Notably, the highest sulfate reduction rates were observed from Needles samples, which were ~20-fold higher than those observed at Dead Dog, and ~2-fold greater than at Chowder Hill. Many of the rates exhibit large deviations due to the high variability among the biological replicates, most likely due to persistent mineralogical and microbiological

heterogeneity across incubations, even after homogenization. Sulfate reduction was also observed in molybdate amended experiments, though we suspect that molybdate was scavenged by minerals that attenuated the effect of the inhibitor as has been previously observed in metal-rich environments (Bostick *et al*. 2003; Xu *et al*. 2006).

Quantification of Taxonomic and Functional Genes

The abundance of total bacteria, archaea (16S rRNA genes), sulfate reducing prokaryotes (*aprA* gene*)* and sulfate reducing bacteria (*dsrA* and *Deltaproteobacteria* specific 16S rRNA genes) were investigated in each deposit by quantitative PCR (Figure 2). Microbial density (as estimated by 16S rRNA gene copies g^{-1} mineral) was greatest at Needles and lowest at Dead Dog. Microbial communities at each site were dominated by archaea (Figure 2A), with Needles showing the highest ratio of archaea to bacteria (227:1 as compared to 14:1 at Dead Dog or 17.5:1 at Chowder Hill). Assuming an average of 4.19 copies of 16S rRNA gene per bacterium and 1.71 copies of 16S rRNA gene per archaeon genome (Lee *et al*. 2009; Klappenbach *et al*. 2001), Needles hosts a microbial community of 4.12×10^8 cells g^{-1} sample, 3 orders of magnitude higher than Chowder Hill (8.96×10^5 cells g^{-1} sample) and Dead Dog (5.65×10^5 cells g^{-1} sample).

16S rRNA gene primers specifically targeting ribotypes allied to *Desulfovibrio, Desulfomicrobium, Desulfuromusa*, and *Desulfuromonas* were used to enumerate *Deltaproteobacteria* known to mediate sulfate reduction in many marine systems (Stults *et al.* 2001). However, given the difficulty in amplifying 16S rRNA genes from deep-sea thermophiles with typical primer sets - due to mismatches with limited sequence representation in GenBank - it is possible that these assays similarly underestimate abundances in these environments

(Teske & Sorensen, 2008). *Deltaproteobacterial* abundance at Needles was approximately 4.48 x 10^5 copies g^{-1} sample (approximately 26% of the entire bacterial population), though none were detected at Chowder Hill or Dead Dog (data not shown). The abundance of both functional genes for sulfate reduction, *dsrA* and *aprA*, was greatest at Needles and lowest at Dead Dog (a pattern similar to that seen in the 16S rRNA gene abundance estimates; Figure 2B). If we assume an average of 1 *dsrA* gene copy per genome (Klein *et al*. 2001; Kondo *et al*. 2004), the proportion of sulfate reducing bacteria in the bacterial populations is only 2.7% in Needles as compared with 28% in Dead Dog and 53% at Chowder Hill.

Microbial Diversity

454 pyrotag sequencing (bacterial V1-V3 and archaeal V3-V4 of the 16S rRNA gene), rarefaction analyses, and diversity metrics all revealed measureable differences in microbial community composition among the three hydrothermal deposits (Figure 3, Figure 4, Table 3). Via these assessments, Needles hosts the least diverse assemblage of bacteria and archaea, while Chowder Hill and Dead Dog host communities of comparable diversity. Examination of OTUs at 97%, 95% and 92% sequence similarity further reveal differences in microbial community membership among the three sites. Among archaea at the 97% level, only two archaeal OTUs (1% of all archaeal OTUs classified) are shared among the hydrothermal deposits. The sequences classified to these OTUs represent 69%, 48% and 18% of all the library sequences from Needles, Dead Dog and Chowder Hill respectively. One of these OTUs is allied to the ammonium oxidizing archaeal Candidatus *Cenarchaeum* in the phylum *Thaumarchaeota*, and accounts for 35% of Needles and less than 5.0% of Dead Dog or Chowder Hill library sequences. The other OTU is allied to a thermophilic sulfur respiring archaeon within the class

Thermoplasmata. Nearly 40% of the archaeal sequences from Dead Dog were allied to this archaeon. Methanogens allied to *Methanocaldococus* comprised about 1.0% of the total archaeal sequences from Dead Dog, and were not represented in the libraries from Chowder Hill or Needles. Most of the archaeal diversity at Chowder Hill (80% of sequences) and Dead Dog (50% of sequences) was unclassified. No sequences allied to true sulfate reducing archaeal lineages such as *Archaeoglobus fugilis or Aciduliprofundus boonei* were recovered. However, the potential diversity of thermophilic sulfate reducing archaea in these samples is likely much greater than suggested here. This may be explained in part by biases underlying DNA extractions, primer binding and sequencing. For example the archaeal sequencing primers used in this study only target about one third (34%, as assayed by Probe Match; Cole et al. 2005) of the *Archaeoglous*-like sequences contained in the RDP database. Furthermore, the primers may miss members of the dominant *Thermoplasmatales* as *in silico* analysis only returns 48% (1715/3558 sequences) of the RDP reported sequences. In total, these archaeal sequencing primers (349F-806R) miss 42% of the total archaeal sequences (67713/117373 sequences) in the RDP database. Similar bias has been reported in other studies in the deep sea and deep subsurface biotopes (e.g. Dhillion et al., 2003, 2005, Teske et al. 2007).

Among bacteria at the 97% similarity level, 54 of the bacterial OTUs classified (7.0%) were shared among all hydrothermal deposits, and account for 84%, 80%, 71% of the sequences from Chowder Hill, Needles and Dead Dog respectively (Figure4). One of these OTUs accounted for 44%, 36% and 25% of the sequences from Chowder Hill, Dead Dog and Needles respectively. Aligning representative sequences from this OTU via Blast-n (Altschul *et al.* 1997) reveals a best match to *Thermodesulfovibrio*, an anaerobic, thermophilic, sulfate-reducing

299 bacterium, from the phylum *Nitrospira* (81% identity). Given its abundance, we postulate that it
300 likely contributes substantially to the high thermophilic sulfate reduction rates. Furthermore,
301 the *dsrB* gene library was dominated by sequences phylogenetically allied to
302 *Thermodesulfovibrio* (Supplemental Table S1). Other dominant groups of bacteria include
303 members of the *Gammaproteobacteria, Bacteroidetes, Deltaproteobacteria* and *α-*
304 *proteobacteria*. Via Blast-n, most of the unclassified sequences matched to partial 16S rRNA
305 gene sequences from hydrothermal vent fluid communities (Nunoura *et al*. 2010; Sylvan *et al*.
306 2012). Sequences classified as *Deltaproteobacteria* comprised 5.5%, 8.2%, or 14%, of the total
307 population of Chowder Hill, Needles, or Dead Dog respectively. While Dead Dog may have had
308 the highest proportion of its amplicons classified as *Deltaproteobacteria*, sequences related to
309 known sulfate reducers within the *Deltaproteobacteria (Desulfobacteraceae, Desulfobulbus*
310 *rhabdoformis, Desulfovibrio)* were only found at Needles and comprised 1.1% of the 16S rRNA
311 gene library. The majority of the sequences classified as *Deltaproteobacteria* in each of the
312 three sites were from one unclassified *Deltaproteobacterial* OTU consisting of 4.4, 5.3, and 13%
313 of the sequences from Chowder Hill, Needles and Dead Dog respectively.

314 **DISCUSSION**

315 Sulfate reduction rates measured in deposits recovered from the Middle Valley vent
316 field reveal the potential for active sulfate reduction within hydrothermal deposits. The
317 magnitude of all measured rates (from 15.7 nmol g^{-1} day^{-1} at Dead Dog at 60°C to 2670 nmol g^{-1}
318 day^{-1} at Needles at 90°C) under experimental conditions were markedly higher than those
319 typically observed in non hydrothermal deep sea sediments (0.1-10 nmol g^{-1} day^{-1}, converted
320 here for comparison assuming an average sediment density of 2 g cm^{-3}; Elsgaard, Isaksen, *et al*.

1994; Weber & Jorgensen, 2002; Joye *et al*. 2004). These rates are comparable in magnitude to those previously observed in hydrothermally influenced sediments (eg. Guaymas basin or Lake Tanganyika (Weber & Jorgensen, 2002; Kallmeyer & Boetius, 2004; Elsgaard, Isaksen, *et al*. 1994; Elsgaard, Prieur, *et al*. 1994), even though the availability of organic carbon is markedly higher in these hydrothermal vent sediments, with Guaymas having up to 200-times greater concentrations of organic carbon (Lang *et al*. 2006; Cruse & Seewald, 2006; Chen *et al*. 1993). To date, the only other measurement of sulfate reduction from sulfide deposits along the East Pacific Rise exhibited rates comparable to those reported here, (Bonch-Osmolovskaya *et al*. 2011), but it should be noted that their samples were incubated under pure H_2 atmosphere.

Notably, the maximum rates of sulfate reduction in Middle Valley sulfides occurred at 90°C in all three deposits. This is in contrast to measurements of sulfate reduction in hydrothermal sediments, where the greatest rates are often observed between 40-70°C, and more modest rates of sulfate reduction have been reported between 80-91°C (Weber & Jorgensen, 2002; Elsgaard, Prieur, *et al*. 1994; Elsgaard, Isaksen, *et al*. 1994). The relatively low or insignificant sulfate reduction rates between 4-80°C suggest Middle Valley deposits harbor a high proportion of hyperthermophilic sulfate-reducing microbes.

The significant differences in the rates we observed among deposits (Kruskal-Wallis, $p<0.0001$) are likely due to differences in biomass and the composition of microbial communities that are influenced by the geochemistry of each deposit. Indeed, microbial biomass (as estimated by 16S rRNA genes) directly correlates to rates of activity and is likely one of the strongest factors affecting the observed rates of sulfate reduction (Pearson correlation coefficient $r=0.879$, $p<0.0005$). Needles had both the highest observed rates as well

as the highest cell density (Figure 2 and 3). Of all the deposits sampled, Needles had the lowest venting fluid temperature (123°C) resulting in the largest zone of microbial habitability. Consistent with this, Needles also had the greatest abundance of *dsrA* and *aprA* genes per gram, suggesting a larger potential sulfate reducing community. Here, *Deltaproteobacteria* allied to *Desulfovibrio, Desulfobulbus, Desulfobacteria*, and *Desulfuromonas* account for 25.7% of the bacterial community. These clades of *Deltaproteobacteria* were not observed at Chowder Hill or Dead Dog by either qPCR enumeration or pyrosequencing. Cultured representatives from some of these *Deltaproteobacterial* clades (*Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans)* have been shown to reduce sulfate at high rates (ranging from 10-1340 nmol min^{-1} mg^{-1} protein) with varying electron donors (Fitz & Cypionka, 1991; Cypionka & Konstanz, 1989).

Thermodesulfovibrio-like organisms dominated the bacterial communities within each hydrothermal deposit (35-44%; Fig. 4). *Thermodesulfovibrio* sp. are considered obligately anaerobic, thermophilic bacteria that can reduce sulfate and other sulfur compounds (Garrity & Holt, 2001). In pure cultures, members of this genus are able to link growth with hydrogen and a limited range of organic carbon molecules (formate, pyruvate and lactate), maintaining optimal growth between 55-70°C (Sekiguchi *et al*., 2008). Needles has a greater proportion of sequences (from pyrosequencing) related to *Thermodesulfovibrio-like* species than the other two deposits. The combination of many sequences related to a thermophilic sulfate reducing bacteria and higher rates of sulfate reduction at elevated temperatures and the, suggests that *Thermodesulfovibrio*-like organisms may play a role in sulfate reduction in warmer environments. However, constraining the relative proportion of sulfate reduction by

365 *Thermodesulfovibrio*-like organisms in these mixed communities was beyond the scope of this
366 study.

367 It is unclear why Chowder Hill and Dead Dog exhibit large differences in rates of sulfate
368 reduction despite other similarities in geochemistry and biomass. One plausible explanation
369 might be that different types of biological interactions (e.g. syntrophy or competition) occur
370 due to differences in the composition and distribution of microbial communities within the
371 mineral matrix of each deposit. Slight differences in community composition, like Dead Dog
372 having a higher representation of sequences related to sulfur respiring (*Thermoplasmata)* and
373 methanogenic (*Methanocaldococus-like*) archaea than Chowder Hill, may lead to biological
374 interactions that have different implications for rates of sulfate reduction in each deposit. Also,
375 substrate competition for H_2 or consumption of locally produced DOC (Oremland & Polcin,
376 1982; Lovley & Phillips, 1987) may be more prevalent in one deposit over another. Future
377 experiments should aim to better resolve how specific interaction between populations, for
378 example, syntrophy or competition for a common substrate, may influence sulfate reduction.

379 ***The relevance of heterotrophic sulfate reduction on hydrothermal vent biomass production***
380 ***and biogeochemistry***

381 Heterotrophic sulfate reduction is likely a prominent metabolic mode within Middle
382 Valley sulfides and sediments, and the sulfate reduction rate data herein (which solely measure
383 hetrotrophic sulfate reduction) support that supposition. Sedimented vent fields typically
384 contain allochthonous organic carbon that could readily support heterotrophy. Indeed, at
385 Middle Valley, bottom waters contain 3.5 mg DOC/L (about 7 fold higher than the overlying
386 surface seawater), while porewater concentrations range from 0.1 – 84.0 mg DOC/L at

sediment depths to 200 mbsf (Ran & Simoneit, 1994). Based on data from culture studies of *Desulfovibrio* strains, including the H^+/H_2 ratio of 1.0 for *Desulfovibrio vulgarius* Marburg (Fitz & Cypionka, 1991), the $P/2e^-$ ratio (number of ATPs produced for every 2 electrons transferred to an electron acceptor) of 1/3 for *Desulfovibrio gigas* (Barton et al. 1983), and the assumption that 10% of ATP production supports growth (20 mmol ATP per gram biomass), our estimates suggest that - at our maximum empirically measured rates - heterotrophic sulfate reduction could support 140 g biomass yr^{-1} (~1.5×10^{14} cells) at Chowder Hill (volume = 109,900 cm^3), 16 g biomass yr^{-1} (~1.7×10^{13} cells) at Needles (volume = 5495 cm^3), and 2.1 g biomass yr^{-1} (~2.2×10^{12} cells) at Dead Dog (volume=12560 cm^3). While these values may be small in comparison to global estimates of chemoautotrophic biomass production on the global ridge system (10^{10} - 10^{13} g of biomass yr^{-1} ;McCollom & Shock 1997; Bach & Edwards 2003), the sulfide produced by these heterotrophic sulfate reducers could represent up to 3% of the H_2S flux from Middle Valley deposits (given previously published vent fluid flow rates from the Main Endeavor field, (Wankel *et al*. 2011)). Additional rate measurements that represent the diversity of physico-chemical conditions found within deposits or ridge systems are necessary to better constrain the contribution of heterotrophic sulfate reducers to global vent biomass and geochemistry.

Hydrothermal vents are dynamic environments where carbon and sulfur cycling are intimately linked. Both autotrophic and heterotrophic sulfate reducing microbes have been isolated from vents, and the data shown here are among the first to constrain the potential for heterotorophic sulfate reduction at vents (in particular those with higher organic carbon loads), as well as the relationship between sulfate reduction rates, temperature, microbial biomass and community density and composition. These data, as well as the vent field estimates of

sulfate reduction, , underscore the relevance of sulfate reduction in hydrothermal ecosystems and further indicate the need for continued studies of sulfur cycling along ridge systems.

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Supplementary information is available at ISME's website

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